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Prostate Cancer is a significant cancer in elderly men. In year 2000 nearly 200,000 cases were diagnosed. Unfortunately over 40,000 patients succumbed to this horrendous cancer. Death is preceded by a characteristic triad of diffuse osteoblastic-osteosclerotic skeletal metastases, bone pain and pathologic fractures. A great majority of prostate cancers metastasize to bone, especially spine and pelvis, and cause pathologic fractures due to osteosclerosis. Bone Morphogenetic Proteins (BMPs) are critical signaling molecules in new bone formation and are expressed in prostate. This work investigates BMP receptors and BMP antagonists to understand the basic mechanisms to inhibit the BMP signaling in metastatic cancer leading to bone pain and pathologic fractures. We investigated the influence of DAN and Noggin, BMP antagonists, on BMP-induced bone formation. The accumulated knowledge will aid in the design of new drugs to reduce and treat pathologic fractures and improve quality of life for patients with prostate cancer.

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# Contents

Cover . . . . .	1
Contents . . . . .	2
Form SF298 . . . . .	3
Introduction . . . . .	4
Body . . . . .	5
Specific Aim 1 . . . . .	5
Specific Aim 2 . . . . .	7
Specific Aim 3 . . . . .	9
Additional Findings . . . . .	9
Key Accomplishments . . . . .	11
Reportable Outcomes . . . . .	12
Conclusions . . . . .	12
References . . . . .	12
Appendices . . . . .	13

## Introduction

Prostate cancer was diagnosed in approximately 200,000 Americans and caused nearly 25,000 deaths last year. Excruciating bone pain, fractures and death caused by prostate cancer invariably result from metastases, the majority of which are osteoblastic and osteosclerotic metastases to bone. These pathological events are well established, yet there is still very little known about the molecular events involved in this deadly process. During embryonic development, bone morphogenetic proteins (BMPs) act as morphogens to determine cell fate and tissue differentiation, and can induce new bone formation when implanted subcutaneously into rats. BMPs function to recruit progenitor stem cells and initiate their growth and differentiation into bone in a sequence of events reminiscent of the steps seen both in bone formation during embryogenesis, and prostate cancer metastases to bone. BMP functions can be inhibited by antagonists such as Noggin or DAN. DAN is a protein which was originally isolated as a tumor suppressor and is a member of a family of secreted BMP antagonists which function by binding to BMPs and preventing their interactions with BMP receptors. **The underlying hypothesis of this proposal is that BMP morphogen activity is involved with the osteoblastic and osteosclerotic pathology that is commonly associated with prostate cancer skeletal metastases.** Inappropriately high BMP activity can be the result of a combination of several factors, including 1) elevated expression of BMPs by prostate cancer cells, 2) increased signal transduction by BMP receptors and Smads, and 3) decreased expression of BMP antagonists such as DAN or Noggin, and 4) further elevation of BMP expression through autocrine or paracrine loops resulting in a vicious cycle of elevated expression. **The overall objective of this proposal was to understand BMP signaling in prostate cancer and metastases, with an initial focus on BMP6 and BMP-antagonist DAN.**

## Body

### Specific Aim 1

**Goals:** The goal of Specific Aim 1 is the Characterization of mRNA expression levels of BMPs, BMP receptors, Smads, and BMP antagonists in prostate and prostate cancer. The proposed method is by real-time quantitative RT-PCR, using TaqMan chemistry and instrumentation. Expected results are an understanding of which BMP signaling pathways may be actively used in prostate and prostate cancer.

The technique used for this initially was quantitative RT-PCR using the TaqMan chemistry. Currently we have primers available to assay human BMPs 2, 3, 4, 6, 7, 8, 11 and 14, BMP receptors type IA, IB, and type II, Smads 1, 5, 6 and 7, and BMP antagonists Cerberus, DAN, Chordin, Gremlin, and Noggin, and the house-keeping mRNAs  $\beta$ -actin or GAPDH. Values were normalized to the levels of GAPDH or  $\beta$ -actin for comparison between samples.

**Progress:** To detect the expression levels of BMP morphogens, TaqMan real-time quantitative RT-PCR assays were performed on total RNA isolated from five different human prostate cancer biopsies, and from the human prostate cancer cell lines LNCaP, DU-145, and PC-3. In cancer biopsies, we found that the most highly expressed member of the BMP family was BMP-6. Messenger RNAs for the other BMP family members were also detected using the highly sensitive RT-PCR technique, although at levels substantially lower than BMP-6. We also detected the expression of all BMPs tested in the three prostate cancer cell lines, although the level of BMP expression in the cell lines did not necessarily match the average levels found in patient biopsies.

To detect the expression levels of BMP antagonists, TaqMan real-time quantitative RT-PCR assays were performed on total RNA

isolated from the LNCaP human prostate cancer cell line. We found expression of DAN, Gremlin, Chordin, Cerberus and Noggin BMP antagonists, listed in order of decreasing level of expression.

The recent advances in microarray technology, specifically in the areas of sensitivity and annotation to the human genome databases, has led us to believe that a microarray based technique would be equivalent to TaqMan in testing for the expression of BMP signal transduction pathway components. The added advantage to the microarray technology is that it profiles the expression levels of many genes in addition to the BMP signal transduction pathway components. Using microarrays, we have profiled the expression of the DU-145 prostate cancer cell line. We identified the presence of transcripts for BMP receptors and Smads, in addition to confirming the expression of BMP mRNAs that we tested using TaqMan technology.

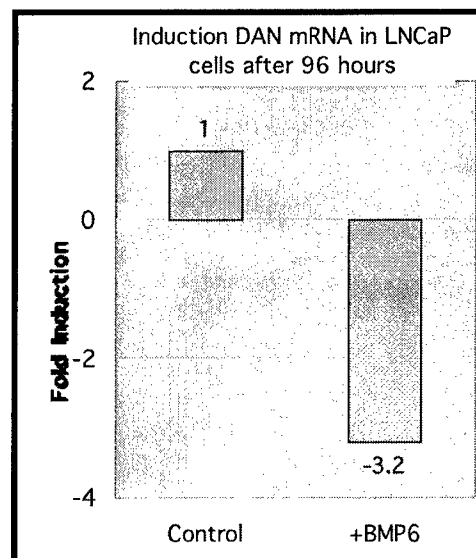


Figure 1: *BMP-6 reduces DAN mRNA expression in LNCaP prostate cancer cell line*

In the preliminary results submitted with this grant application, we showed that BMP-6 treatment of prostate cancer cell lines results in weakly reduced expression of mRNA coding for DAN, a BMP antagonist, as shown in figure 1.

As a follow-up to this observation, we have tested the whether BMP-6 could alter the expression of other BMP antagonists, including Noggin, Chordin, Cerberus and Gremlin. We found that BMP-6 strongly induced expression of Noggin mRNA in a dose-dependent manner, as shown in figure 2. We tested for

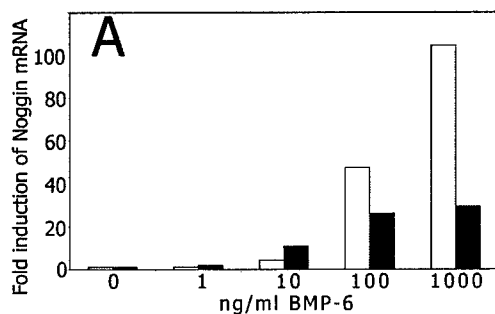


Figure 2: *BMP-6 induces Noggin mRNA in DU-145 (open bars) and LNCaP (filled bars) cell lines*

the production of Noggin protein in DU-145 cell lines in the presence and absence of BMP-6 in the culture medium, and found detectable amounts of Noggin protein only when BMP-6 was added to the culture medium. A western blot of human prostate cancer biopsy homogenate showed that both BMP-6 and Noggin are present at the protein level in the biopsy. These results are shown in figure 3.

Having shown the up-regulation of Noggin mRNA and Protein by BMP-6, we wanted to address the question of whether Noggin could result in the inhibition of BMP-6 activity. While Noggin had been shown to inhibit BMP-2/4 and bind to BMP-7, at the time this work was being performed there was little data available on the ability of Noggin to bind to BMP-6 or inhibit BMP-6 activity.

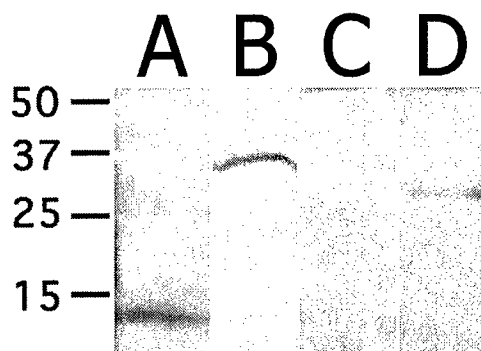


Figure 3: *A human prostate biopsy immunoblotted with anti-BMP-6 (A) or anti-Noggin (B). Cell lysate after growth without BMP-6 (C) and with BMP-6 (D), immunoblotted with anti-Noggin antibody.*

By adding increasing amounts of recombinant human Noggin protein along with a constant 100ng/ml of BMP-6, we were able to show a dose-dependent inhibition of BMP-6 activity in DU-145 cell culture. We found that 3 $\mu$ g of Noggin will completely inhibit the activity of 100ng of BMP-6 in our assay. While this does not formally show a direct binding interaction between Noggin and BMP-6, it is highly suggestive of such an interaction. Evidence for this is that direct binding of Noggin to closely-related BMP-7 was shown to be the mechanism of inhibition.

We have shown that BMP-6 and Noggin are present in homogenized prostate biopsies at the protein level, that BMP-6 will induce Noggin production in cells, and that Noggin can inhibit BMP-6 activity. To gain insight of whether these proteins might be interacting with each other in the prostate tissue, we performed immunohistochemistry to examine the distribution of noggin in normal and cancerous human prostates. All prostate biopsies reacted positively with the anti-Noggin antibody. Staining in normal prostate was observed primarily in epithelial cells, with little or no staining in the stromal areas. In high

grade cancers, Noggin protein was localized to areas dense with carcinoma cells. In samples with mixed areas of connective tissue and carcinoma, there was also little or no staining in the connective tissue. The distribution of Noggin appears very similar to the distribution of BMP-6 shown by others, indicating the possibility of an interaction between these proteins in the prostate tissues.

The results from this specific aim have been published in Cancer Research, Issue 64 pages 8276 through 8284, on November 15, 2004.

## Specific Aim 2

**Goals:** The goals of Specific Aim 2 were to generate sub-clones of LNCaP cell lines that over-express BMP6, and sub-clones that over-express BMP antagonist DAN, by stable plasmid transfections. Additional goals were to test the hypothesis that recombinant BMP-6 can bind to recombinant DAN *in vitro*, and if so, test the hypothesis that DAN can inhibit the BMP-6 bone-forming activity *in vivo* using our established bio-assay.

**Progress:** We cloned BMP-6 and DAN cDNAs individually into the pcDNA6 mammalian expression vector sold by Invitrogen, and then transform LNCaP or PC-3 prostate cancer cells. The transformed cell populations were placed under selective pressure with Blasticidin for three weeks to eliminate untransformed cells. This was accomplished. However, when the Blasticidin resistant cells (containing the pcDNA6 plasmid) were tested for the production either BMP-6 or DAN, we were unable to detect increased levels of either protein. The pcDNA6 vector uses the constitutively strong CMV promotor to drive the expression of the cloned cDNA. We believe that there may be selective pressures against cells which constantly produce such unnaturally high amounts of BMP-6 or DAN protein, and therefore that the cells surviving the three week Blasticidin selection process have lost the ability to express BMP-6 or DAN.

To overcome these technical obstacles, we decided that an inducible expression system would be more appropriate, and decided on the Tet-ON retroviral system manufactured by Clontech. In this system, cells are first permanently transfected with a Tetracycline regulatory element. This element (rtTA) will activate expression of cDNAs cloned downstream of a Tetracycline Response Element (TRE) in the presence of Doxycycline, a synthetic analog of tetracycline. In the absence of Doxycycline, transcription of the cDNA is highly repressed.

We have succeeded in generating several subclones of the LNCaP and DU-145 prostate cancer cell lines which permanently express the Tet-ON regulatory element. These cell lines are a valuable research tool, with the potential to change not only the level of BMP-6 or DAN that is produced, but also the time during which the protein is produced, simply by adding or removing varying amounts of Doxycycline.

Using a luciferase reporter, we have quantified the fold-induction and basal expression levels in these cells. There is a low basal level of luciferase expression even in the absence of Doxycycline. In the presence of nanogram to microgram amounts of Doxycycline, expression of the luciferase reporter increases in a dose-dependent manner to approximately 10 to 50 times the basal level, depending on the cell line and the individual clone tested. We have cloned the human Noggin cDNA into the appropriate vector for use in this system, and are in the process of cloning DAN and BMP-6 into this vector. We believe that the low basal expression in the absence of Doxycycline will eliminate the selective pressures against the continued over-expression of DAN or BMP-6 that we have observed using the pcDNA6 system, and therefore overcome the technical limitations encountered.

Since the last progress report, we have successfully generated Du145 prostate cancer cell lines that express inducible amounts of Noggin protein under control of the Doxycycline responsive TetON promotor, shown in Figure

4. The basal level of gene expression in this system is still relatively high even in the absence of Doxycycline induction. This is evident from the immuno-reactive band in the 0  $\mu\text{g}/\text{ml}$  lane of Figure 4, which shows noggin protein being produced at detectable levels without induction. Untransfected Du145 cells do not produce detectable levels of Noggin protein using immuno-blot detection. An mRNA analysis of noggin message in these stable cell lines mirrors what is seen at the protein level. TaqMan quantitative RT-PCR analysis shows that there is an increased basal level of Noggin mRNA even in the absence of Doxycycline induction. On top of this high basal level of expression, there is a further 2-3 fold induction possible using 0.1 and 1.0  $\mu\text{g}/\text{ml}$  Doxycycline. At concentrations of doxycycline above 2.0  $\mu\text{g}/\text{ml}$ , further increases in expression were observed although with substantial toxicity to the cells.

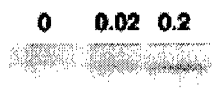


Figure 4: Du145 cells with inducible Noggin protein under control of the TetON promoter. Figure shows a western blot of lysate from cells grown in 0.0, 0.2, and 0.2  $\mu\text{g}/\text{ml}$  Doxycycline, an immunoreactive band at approximately 25kDa is shown.

BMP-6 and Noggin retrovirus were infected into both LNCaP and Du145 cell lines, and antibiotic-resistant, infected cell populations were obtained and frozen in liquid nitrogen for analysis. To date, we have characterized only the inducible Noggin producing Du145 cells, as shown in Figure 4 and discussed on page 8. The complete characterization of the inducible Noggin LNCaP, and inducible BMP-6 cell sub-lines is in progress. At this point, stable integration of the inducible constructs coding for antibiotic resistance alongside BMP-6 or Noggin have been confirmed in both LNCaP and Du145 TetON cells. The

protein production levels of BMP-6 need to be assayed at the basal and doxycycline induced levels in both cell lines.

We have demonstrated direct binding interactions *in vitro* of biotinylated recombinant DAN to BMP-6 and BMP-7, as well as transforming growth factor beta-2 (TGF- $\beta$ 2), as shown in figure 5. There was little or no binding of DAN to the negative control, bovine serum albumin (BSA), indicating that the observed binding is specific to the BMPs and TGF- $\beta$ 2.

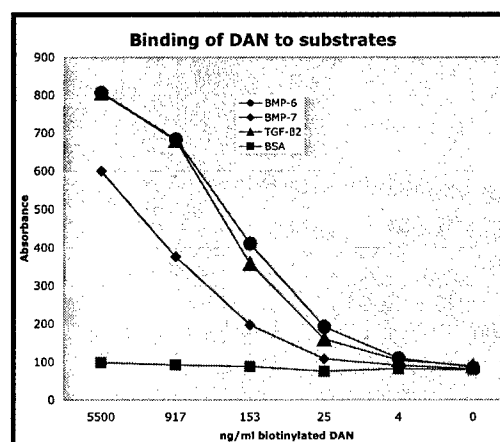


Figure 5: Binding of various dilutions of biotinylated DAN protein to constant amounts of BMP-6, BMP-7, and TGF- $\beta$ 2 or BSA coated onto microplates

Since we have shown the direct binding of DAN to BMP-6, the next task was to test whether DAN could inhibit the activity of BMP-6. These experiments were to be done first in cultures of prostate cancer cell lines, later in an *in vivo* bio-assay. Unfortunately the recombinant DAN that we were able to produce in the *pichia pastoris* yeast expression system was not of sufficient quality for use in cell culture. We believe that non-protein contaminants in our preparations negatively affected cell viability when DAN was added to cell cultures in the amounts required to inhibit BMP-6 activity. However, recombinant human DAN protein has recently be-



come available commercially, and we expect to be able to use the commercial product for the BMP-6 inhibition studies in cell culture and in the *in vivo* bioassays.

### Specific Aim 3

**Goals:** Use an established SCID (severe combined immunodeficient) mouse model of cancer metastasis to directly test the hypothesis that over-expression of BMP-6 or DAN affects the pathology of bone metastases of LNCaP cells, using cell lines generated above. Characterize their behavior with regards to the kinetics of bone colonization, growth, and the effects on surrounding bone. Identify the BMP signaling pathways active in the injected cells (autocrine effect), and those active in the bone surrounding the prostate cancer lesions (paracrine effects). Determine the androgen dependence of this metastasis model system.

**Progress:** We were somewhat delayed with these animal studies because of the unanticipated anti-proliferative effects of BMP-6 on the prostate cancer cell lines. We were unable to get stable cells that proliferate and also constitutively produce BMP-6, and therefore unable to use BMP overproducing cells the proposed animal model. We had to re-evaluate our strategy and decided to create cell lines that have a Tet-ON control element, which is used to drive expression of BMP-6 (or Noggin or DAN) in an inducible manner upon addition of the tetracycline analog Doxycycline. The first step was to create LNCaP and Du145 prostate cancer cells stably expressing the TetON regulatory elements. This was accomplished in 2003: we created stable LNCaP and Du145 cells with the TetON regulatory elements. The next step involved titration of the Doxycycline response curve with these cells, using a luciferase reporter system. This was accomplished in early 2004: we showed inducible expression of luciferase in both cell types. The final step was to introduce retroviral constructs coding for BMP-6, and BMP antagonist DAN or Noggin into these cells.

We have accomplished this step for BMP-6 and Noggin, but not for DAN. The final step is to characterize the level of inducible expression of BMP-6, Noggin and DAN in these cells. We have accomplished this step for Noggin in Du145 cells, but not yet for BMP-6 or DAN in LNCaP cells. Once this is finished, the cell lines will be characterized and ready to use in the proposed animal model of prostate cancer metastasis to bone.

The negative selective pressure of BMP-6 and DAN overexpression proved to be a setback in the time-scale of this project, and necessitated the revised strategy for specific aims 2 and 3 discussed in the preceding paragraph. However, there are great advantages to the inducible expression system. This inducible system will allow us to control not only the level of BMP-6, noggin or DAN that is produced, but also the time during which the proteins are produced, simply by adding or removing varying amounts of Doxycycline.

### Additional Findings

We were initially disappointed by the inability to clone stable cell lines with constitutive over-expression of BMP-6 as proposed in Specific Aim 2. To investigate whether we may have introduced negative selective pressure or other deleterious effects through constitutive expression of BMP-6, we examined the response of Du145 and LNCaP cells to exogenously added BMP-6. The following knowledge resulted from this line of experiments:

BMP-6 signals in LNCaP and Du145 cells through phosphorylation of Smad 1, 5, and 8 proteins. The phosphorylation of Smads 1/5/8 is detected within 15 minutes of BMP-6 treatment, and out to at least 96 hours of continuous BMP-6 treatment. This increase in Smad phosphorylation occurs with little or no change in the total Smad protein within the time period studied.

BMP-6 induces expression of p21 protein, a negative regulator of cell cycle progression. In many situations, p21 expression is dependent on signals from the p53 pathway. We

show that p21 is up-regulated by BMP-6 in both LNCaP and Du145 cells, which is notable because Du145 cells have a deficient p53 pathway. We demonstrated that p21 is induced by BMP-6 within 90 minutes and out to at least 96 hours of continuous BMP-6 treatment.

Since p21 is a negative regulator of cell cycle progression induced by BMP-6, we studied the proliferation of LNCaP cells in the presence and absence of BMP-6. We showed that in serum-deprived conditions, BMP-6 is a negative regulator of cell growth, and that this growth inhibition is independent of androgens.

The negative regulation of cell growth by BMP-6 is one plausible explanation for the difficulties encountered in generating stable cells that constitutively over-express BMP-6, but there may be others.

To comprehensively examine other cellular responses to BMP-6, we performed microarray analysis of BMP-6 treated versus untreated Du145 cells. Affymetrix U133A 2.0 Plus genes were used, these chips have each over 47,000 individual probes representing all or most of the known human genes. This information was used to tabulate the expression of every protein known to be involved in BMP signaling, including all BMP family member, all extracellular antagonists, all intracellular signal-transduction molecules, and many more genes which had been reported to interact with the BMP pathway at some level. This significantly expands on the accomplishments of Specific Aim 1, and this work was included in the Cancer Research publication of 2004 included in the appendix 3.

The comprehensive list of BMP-6 response genes described above was generated from an *in vitro* treatment of prostate cancer cells with BMP-6. Since elevated BMP-6 expression correlates with worse cancer prognosis, we decided to cross-reference our BMP-6 response genes with "Cancer Signature Genes" identified by others (Febbo and Singh et al in Cancer Cell, 2002 as well as other public cancer gene databases). We found that a significant

15% of the top 100 "Cancer Signature Genes" were under control of BMP-6 in our simplified *in vitro* system. This provides further evidence of a causative role for BMP-6 in cancer progression.

## Key Accomplishments

- Specific Aim 1

- Characterized mRNA levels of BMPs 2, 3, 4, 6, 7, 8, 11, 14 expression in prostate cancer biopsies
- Characterized mRNA levels of BMPs 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, BMP receptors, Smads, and BMP antagonists Noggin, Dan, Chordin, Cerberus and Gremlin in a prostate cancer cell line
- Found Dan (a BMP antagonist) to be weakly down-regulated by BMP-6 protein in prostate cancer cell lines
- Found BMP-6 mRNA to be highly expressed in prostate cancer biopsies and in cell lines
- Found BMP-6 protein to be present in prostate biopsies
- Found Noggin (a BMP antagonist) mRNA and Protein in prostate biopsies and cell lines
- Found BMP-6 protein to strongly up-regulate levels Noggin mRNA expression and Protein production in prostate cancer cell lines
- Demonstrated that Noggin inhibits BMP-6 function in a dose-dependent manner
- Showed Noggin distribution in epithelial cells but not stromal areas of normal and cancerous prostate
- Performed microarray analysis to categorize expression of over 47,000 genes produced by Du145 prostate cancer cells
- Determined gene expression response of Du145 cells to BMP-6 treatment
- Correlated BMP-6 response genes in Du-145 cells to “Prostate Cancer Signature Genes” identified by Febbo and Singh *et al* in Cancer Cell. 1: 1-7 (2002). Found 13 of top 100 “Signature Genes” controlled by BMP-6 in Du145 cells
- Compiled a complete list of BMP signal transduction proteins expressed in prostate, prostate cancer, and Du145 prostate cancer cells

- Specific Aim 2

- Generated stable subclones of LNCaP and DU-145 cell lines with Tet-ON regulatory element for inducible expression of recombinant proteins
- Cloned Noggin into retroviral expression vector for use in Tet-ON cells lines
- Generated and characterized stable sub-lines of Du145 TetON cells with demonstrated Doxycycline-inducible Noggin expression
- Generated (uncharacterized) sub-lines of LNCaP TetON cells with potential for producing Doxycycline-inducible Noggin expression
- Generated (uncharacterized) sub-lines of Du145 and LNCaP with potential for producing Doxycycline-inducible BMP-6 expression
- Demonstrated direct binding of recombinant DAN protein to BMP-6, BMP-7, and TGF- $\beta$ 2.

- Specific Aim 3
  - Use of inducible expression system (Aim 2) will allow tight control of expression level and timing of expression in proposed animal model
- Additional Findings
  - Demonstrated that BMP-6 signals through phosphorylation of Smad1/5/8 in prostate cancer cells
  - Showed that BMP-6 induces p21 expression in LNCaP cells and in Du145 cells (which have a compromised p53 pathway)
  - Found that BMP-6 inhibits proliferation of Du145 and LNCaP cells in serum-deprived conditions, in an androgen-independent manner
  - Characterized BMP-6 response genes by microarray analysis, examined over 47,000 different transcripts and identified those regulated by BMP-6 in timecourse experiments

## Reportable Outcomes

- Manuscript published in Cancer Research 64:8276-84, 2004 (see appendix)
- Presentation of results in Cancer Center Seminar Series at UC Davis Medical Center
- Presentation of results at UC Davis Cancer Center 10th annual Cancer Research Symposium
- Presentation of results in Biomedical Engineering Seminar Series at UC Davis
- Completion of Ph.D. Dissertation by Dominik Haudenschild
- Development of stable subclones of LNCaP and Du-145 cells with Tet-ON regulatory elements
- Development of stable subclones of Du145 Tet-ON cells with inducible Noggin expression

## Conclusions

We have successfully completed the work proposed in specific aim 1 and 2, and are prepared to complete specific aim 3. The accrued knowledge of BMP signaling pathways, including receptors and native antagonists, and BMP-response genes, will help aid in the design of potential antagonists to block BMP signaling and reduce pain and pathologic fractures of bone in prostate cancer metastases.

## **Appendices**

Publication in Cancer Research 64:8276-84, November 15, 2004

“Bone Morphogenetic Protein (BMP-6) Signaling and BMP Antagonist Noggin in Prostate Cancer”

# Bone Morphogenetic Protein (BMP)-6 Signaling and BMP Antagonist Noggin in Prostate Cancer

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## ABSTRACT

It has been proposed that the osteoblastic nature of prostate cancer skeletal metastases is due in part to elevated activity of bone morphogenetic proteins (BMPs). BMPs are osteoinductive morphogens, and elevated expression of BMP-6 correlates with skeletal metastases of prostate cancer. In this study, we investigated the expression levels of BMPs and their modulators in prostate, using microarray analysis of cell cultures and gene expression. Addition of exogenous BMP-6 to DU-145 prostate cancer cell cultures inhibited their growth by up-regulation of several cyclin-dependent kinase inhibitors such as p21/CIP, p18, and p19. Expression of noggin, a BMP antagonist, was significantly up-regulated by BMP-6 by microarray analysis and was confirmed by quantitative reverse transcription-polymerase chain reaction and at the protein level. Noggin protein was present in prostate biopsies and localized to the epithelial components of prostate by immunohistochemistry. Recombinant noggin inhibited the function of BMP-6, suggesting a negative feedback regulation of BMP activity and indicating a strategy for the development of a novel therapeutic target in the treatment of painful osteosclerotic bone metastases of prostate cancer.

## INTRODUCTION

Prostate cancer was diagnosed in more than 220,000 Americans last year, and nearly 30,000 Americans died of this disease. Excruciating bone pain, fractures, and death caused by prostate cancer are invariably the result of metastases, the majority of which are osteoblastic and osteosclerotic to bone (1–5). Whereas the pathological events are well established, the molecular events involved in prostate cancer metastasis are only beginning to be understood. The bone morphogenetic protein (BMP) family of proteins was originally identified by the ability of BMPs to induce new bone formation when implanted in ectopic subcutaneous sites in rats (6–8). BMPs initiate new bone formation by recruiting progenitor stem cells and initiating their growth and differentiation into bone in a sequence of events reminiscent of the steps seen in bone formation during embryogenesis (8). BMPs are active at low concentrations (10–50 ng/ml), although the exact determination of BMP concentrations required to elicit a response *in vivo* is complicated by the fact that BMPs are often bound to the extracellular matrix rather than soluble. At the mRNA level, it has been shown that expression of BMP-7, but not BMP-4, is androgen dependent in mouse (9) and human prostates (10).

The profound effects that BMPs have on cell fate and determination necessitate a tight regulation of BMP activities. There are more than a dozen different human BMPs, each of which has its own subset of activities and unique expression patterns. This regulation can occur at different levels of the BMP signaling cascade, either extracellularly, at the cell membrane, or within the cell. A family of extracellular BMP

antagonists has recently been discovered. These are secreted proteins that bind to BMPs and reduce their bioavailability for interactions with the BMP receptors. Extracellular BMP antagonists include noggin, follistatin, sclerostatin, chordin, DCR, BMPMER, cerberus, gremlin, DAN, and others (refs. 11–16; reviewed in ref. 17). There are several type I and type II receptors that bind to BMPs with different affinities. BMP activity is also regulated at the cell membrane level by receptor antagonists such as BAMBI (18), which acts as a kinase-deficient receptor. Intracellularly, the regulation of BMP activity at the signal transduction level is even more complex. There are inhibitory Smads (Smad-6 and Smad-7), as well as inhibitors of inhibitory Smads (AMSH and Arkadia). There are proteins that can target either receptors or Smads for degradation (Smurf, SANE, and CHIP) and phosphatases that reduce receptor phosphorylation (GADD34-PP1c). A host of receptor and Smad binding proteins can modulate the nuclear translocation of Smad complexes (EID and DLX) or alter Smad binding to the receptors (XIAP, BRAM, FKBP12, and others). Within the nucleus, transcriptional coactivators and corepressors modulate the transcription of BMP response genes (*YY1*, *Ski*, *Sno*, *ECSIT*, *Tob*, *CBP/P300*, and many more). It is the combined effect of this large symphony of BMP signaling molecules that will ultimately determine cellular response to BMP.

BMP-6 expression is increased in prostate adenocarcinoma, and several studies have shown a correlation between elevated BMP-6 and osteoblastic metastases (19–23). At present, there is still much to be learned about the function of BMPs and their antagonists in prostate cancers. In this study, we investigated the expression levels of BMPs, their antagonists, and molecules associated with BMP activity. In particular, we investigated the expression of BMP-6 and the BMP antagonist noggin in prostate cancer cell lines, with special focus on reciprocal feedback regulation and proliferation.

## MATERIALS AND METHODS

**Cell Culture.** DU-145 and LNCaP cells were purchased from American Type Culture Collection (Manassas, VA) and cultured in the recommended media (Invitrogen, Carlsbad, CA). Fetal bovine serum (FBS) and charcoal-stripped FBS were purchased from Hyclone (South Logan, UT). Cells were seeded in media supplemented with 10% FBS at a density of 200,000 cells per well in 6-well dishes (approximately 20,000 cells/cm<sup>2</sup>) and allowed to attach overnight. The culture medium was replaced with media supplemented with 1% charcoal-stripped FBS for 24 hours, and then BMP-6, BMP-7, noggin, and steroid hormones (in media supplemented with 1% charcoal-stripped FBS) were added for the indicated times. Phenol red was not a component of any media used. Recombinant noggin was a generous gift from Dr. Aris Economides (Regeneron Pharmaceuticals, Tarrytown, NY). BMP-6 and BMP-7 were generous gifts from Dr. T. K. Sampath (Creative Biomolecules, Hopkinton, MA). Unless noted, all other reagents were purchased from Invitrogen.

**Quantitative Reverse Transcription-Polymerase Chain Reaction.** DNA-free total RNA was prepared with RNeasy mini-columns, using the on-column DNase digestion step (Qiagen, Valencia, CA). Quantitative reverse transcription-polymerase chain reaction (RT-PCR; TaqMan) primers and probes were designed using Primer Express software (Applied Biosystems, Foster City, CA), and their sequences (in 5' to 3' direction) were as follows: BMP6Fwd, CCGTGTAGTATGGGCTCAGA; BMP6Rev, TCACAACCCACAGATTGCTAGTG; BMP6Probe, CGTGATGTCAAATTCAGCCAGCCTT; NogginFwd, CCTGGTGGACCTCATCGAA; NogginRev, CAGCGTCTCGT-

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TCAGATCCTT; NogginProbe, ACCCAGACCCTATCTTTGACCCAGG; GremlinFwd, GCAAAACCCAGCCGCTTA; GremlinRev, GGTGTGATGAT-GGTGCGACTGT; GremlinProbe, CAGACCATCCACGAGGAAGGCTGC; CerberusFwd, ACCATGAGGAAGCTGAGGAGAA; CerberusRev, GCAGG-GCTGGTGGCTACA; and CerberusProbe, AGATCTGTTTGTGCGAGTGC-CACACC. Real-time quantitative RT-PCR was done using a Sequence Detector 7700 and the EZ-RT-PCR reagents from Applied Biosystems. Expression of each mRNA was normalized to that of glyceraldehyde-3-phosphate dehydrogenase, and the fold change was calculated using the formula  $2^{\Delta\Delta C_t}$  as described by Applied Biosystems. Each RT-PCR was done in triplicate, and the results shown are representative of at least two repeated experiments.

**Western Blotting.** Human prostate cancer biopsies were obtained from Dr. Ralph de Vere White (Department of Urology, University of California Davis Medical Center) and homogenized in reducing SDS-PAGE sample loading buffer that contained 8 mol/L urea. After centrifugation for 5 minutes at  $12,000 \times g$ , the supernatants were boiled for 5 minutes and then electrophoresed on precast 4–12% gradient gels (Invitrogen). Cultures of 200,000 cells were lysed in 100  $\mu$ l of 1 $\times$  SDS-PAGE sample buffer, and 40  $\mu$ l were loaded into each lane of a SDS-PAGE precast cell (corresponding to 80,000 cells per lane for even protein loading), which was then processed as described above. After transfer to Immobilon-P (Millipore, Bedford, MA), the membranes were blocked with 2% bovine serum albumin and then incubated with the anti-noggin (Regeneron Pharmaceuticals) and anti-BMP-6 antibodies at 2 to 10  $\mu$ g/ml and anti-p21 (BD Biosciences, San Jose, CA) and anti-phospho-Smad (Cell Signaling, Beverly, MA) at the dilutions recommended by the manufacturers. Detection was done by either horseradish peroxidase-conjugated goat antirabbit secondary antibodies and enhanced chemiluminescence (ECL; Amersham Biosciences, Piscataway, NJ) or alkaline phosphatase-conjugated goat antibodies and Enhanced ChemiFluorescence and quantified on a Storm-860 scanner (Amersham Biosciences).

**Immunohistochemistry.** A slide array of 80 characterized, paraffin-embedded 2-mm human prostate biopsies (Imgenex, San Diego, CA) was characterized by Imgenex using the Gleason grading scale. The slide array was incubated with 25  $\mu$ g/ml mouse anti-noggin monoclonal antibody using the VectaStain ABC-AP kit according to the manufacturer's protocols (Vector Laboratories, Burlingame, CA).

**Cell Proliferation Assay.** Cells were seeded in media supplemented with 10% FBS at a density of  $1.5 \times 10^5$  cells per well in 6-well dishes and allowed to attach overnight. The culture medium was replaced with media supplemented with 10% charcoal-stripped FBS for 24 hours, and then BMP-6 and dihydrotestosterone [DHT (in media supplemented with 10% charcoal-stripped FBS)] were added for a period of 7 days. Media were replaced and supplemented with BMP-6 and DHT every 2 to 3 days. Cells were lysed by the addition of 500  $\mu$ l 0.5% SDS solution, and DNA was isolated using the phenol/chloroform method. The relative DNA concentration was obtained using the PicoGreen assay (Molecular Probes, Eugene, OR) and quantified on a Storm-860 scanner.

**Microarray Analysis.** DU-145 cells were seeded in media supplemented with 10% FBS at a density of  $3 \times 10^6$  cells per 150-cm<sup>2</sup> flask (approximately 20,000 cells per cm<sup>2</sup>) and allowed to attach overnight. The culture medium was replaced with media supplemented with 1% charcoal-stripped FBS for 24 hours, and then BMP-6 was added in media supplemented with 1% charcoal-stripped FBS. DNA-free total RNA was prepared using RNeasy mini-columns, using the on-column DNase digestion step. RNA samples were processed for analysis on Affymetrix U133 2.0 Plus arrays by the Gene Expression Resource core facility at University of California Davis Medical Center. These arrays cover the entire human genome and include more than 47,000 gene transcripts.

**Public Databases.** Twenty-seven normal and 52 cancerous prostate samples were processed on Affymetrix U95A microarrays by Singh *et al.* (24), and the data were made publicly available.<sup>1</sup> We analyzed these files with dChip 1.3<sup>2</sup> to determine the presence or absence of each gene listed and whether there was differential regulation in tumor *versus* normal biopsies. SAGE and elec-

tronic Northern expression data, when available, were taken from the Cancer Genome Anatomy Project databases.<sup>3</sup>

## RESULTS

It is the combined effect of the many BMP signaling and regulatory proteins that ultimately determines cellular response to the presence of exogenous BMP. We decided on a simple experimental model consisting of DU-145 prostate cancer cells cultured in the presence or absence of exogenously added BMP-6. We used this model to determine which of the BMP signaling genes were (a) present in the cells and (b) affected by the addition of BMP-6. Microarray experiments were performed with RNA harvested from BMP-6-treated and control DU-145 cells. These experiments confirmed the presence of BMP-6 mRNA, as well as the presence of the type I and type II BMP receptors and Smads required for BMP signal transduction. The various components of the BMP signaling system expressed by DU-145 cells are presented in Table 1.

We next extended these findings beyond the culture model and asked which of these BMP signaling genes are present in prostate using publicly available cancer microarrays and serial analysis of gene expression (SAGE) data (Table 1). In the genes detected, there is considerable agreement between the prostate biopsies and the DU-145 cell line. However, comparison of extended BMP family and related signaling molecules showed that many genes that were present in the DU-145 cell line were not detectable in RNA from the prostate biopsies.

We were particularly interested in the genes that negatively regulate BMP signaling because elevated BMP-6 expression correlated with bone metastasis of prostate cancer. Noggin, Smad-6, Smad-7, and BAMBI are negative regulators of BMP signaling whose expression was increased on BMP-6 treatment of DU-145 cells. BAMBI was also up-regulated in cancerous prostates. The regulation of noggin by BMP-6 will be presented in greater detail below.

**BMP-6 and Noggin Are Present in Prostate.** To confirm the presence of BMP-6 and noggin in prostate at the protein level, immunoblots of prostate cancer homogenate were probed with antibodies against human BMP-6 and noggin. Both of these proteins were expressed in the homogenized prostate cancer biopsies (Fig. 1A, Lanes 1 and 2, respectively).

Immunohistochemistry was used to examine the distribution of noggin in normal and cancerous human prostates. All prostate biopsies reacted positively with the anti-noggin antibody, and Fig. 1B shows four representative images. Staining in normal prostate was observed primarily in epithelial cells, with little or no staining in the stromal cells. In high-grade cancers, noggin protein was localized to areas dense with carcinoma cells. In samples with mixed areas of connective tissue and carcinoma, noggin was localized to the carcinoma cells, and there was little or no staining in the connective tissue. Using this technique, we were not able to demonstrate a difference in noggin staining intensity among normal epithelium and low-grade and high-grade cancer cells.

**BMP-6 Induces Noggin Expression.** Recombinant human BMP-6 protein added to cultures of DU-145 and LNCaP prostate cancer cell lines for 48 hours resulted in a dose-dependent increase in noggin mRNA, as shown in Fig. 2A. This confirms the previous observations made in rat osteoblasts (25) that noggin can be induced by BMP-6 in certain cell types. The mRNA levels of two additional extracellular BMP antagonists, gremlin and cerberus, were examined in the same samples and showed no significant changes in the presence of BMP-6 protein (Fig. 2C). In LNCaP prostate cancer cell culture, the BMP-

<sup>1</sup> <http://www.broad.mit.edu/cgi-bin/cancer/datasets.cgi>.

<sup>2</sup> [www.dchip.org](http://www.dchip.org).

<sup>3</sup> <http://cgap.nci.nih.gov/>.

Table 1 *BMP signaling molecules present in prostate*

Affymetrix probe ID		Significant change with BMP treatment in DU-145 cell line	Normal prostate vs. prostate cancer U-95A chip data	SAGE in prostate (tags per 200,000)
<b>BMP antagonists</b>				
<b>extracellular</b>				
231798_at	Noggin	Up		<2
209763_at	Chordin-like	Absent	Absent	
211248_s_at	Chordin variant 3	Absent		
221674_s_at	Chordin variant 1	Absent	Absent	
203592_s_at	Follistatin-like 3	Present no change	Present no change	
207345_at	Follistatin isoform FST317 precursor	Present no change		
204948_s_at	Follistatin isoform FST344 precursor	Absent		
223557_s_at	Transmembrane protein with EGF-like and two follistatin-like domains 2	Absent		
1564511_a_at	Weakly similar to follistatin-related protein precursor	Absent		
221378_at	CER1 cerberus 1 homolog	Absent		<2
220794_at	Related to DAC and cerberus, =prdc	Absent		<2
224811_at	Sclerostin	Present no change		<2
213456_at	Sclerostin-domain-containing-1 = ectodin	Absent	Absent	
218469_at	CKTSF1B1, DRM; IHG-2; GREMLIN	Absent	Absent	
201621_at	DAN; NBL1; NO3; MGC8972; D1S1733E	Present no change	Down	Down
241986_at	BMPER	Absent		
201894_s_at	Decorin	Present no change		Down
<b>BMP/TGF-<math>\beta</math> family</b>				
<b>members</b>				
205574_x_at	BMP-1, isoform 1	Present no change	Absent	Up
206725_x_at	BMP-1, isoform 2	Absent		
202701_at	BMP-1, isoform 3	Absent		
207595_s_at	BMP-1, isoform 4	Present no change		
205289_at	BMP-2	Present no change	Absent	<2
205290_s_at	BMP-2	Absent		
208244_at	BMP-3	Absent	Absent	
206159_at	BMP-3B = GDF10	Absent	Absent	<2
211518_s_at	BMP-4	Absent	Absent	Down
205430_at	BMP-5	Absent	Absent	<2
205431_s_at	BMP-5	Absent	Absent	<2
206176_at	BMP-6	Present no change	Absent	<2
241141_at	BMP-6	Absent	Absent	<2
209590_at	BMP-7	Present no change	Absent	<2
211260_at	BMP-7	Absent	Absent	<2
211259_s_at	BMP-7	Absent	Absent	<2
207866_at	BMP-8	Absent	Absent	<2
207865_s_at	BMP-8	Present no change	Absent	
221136_at	BMP-9 = GDF-2	Absent		<2
208292_at	BMP-10	Absent	Absent	<2
226234_at	BMP-11 = GDF-11	Present no change	Absent	<2
226232_at	BMP-11 = GDF-11	Absent		
216860_s_at	BMP-11 = GDF-11	Present no change		
216854_at	BMP-11 = GDF-11	Absent		
206614_at	BMP-14 = GDF-5 = CDMP-1	Absent	Absent	<2
1555665_at	BMP-14 = GDF-5	Absent		
221332_at	BMP-15	Absent		<2
216514_at	BMPY	Present no change		
216369_at	BMPY	Absent		
206397_x_at	GDF1	Absent	Absent	
229448_at	GDF1	Absent		
220053_at	GDF-3	Absent		<2
207145_at	MSTN myostatin = GDF-8	Absent	Absent	Up
221314_at	GDF-9	Present no change		<2
221576_at	PLAB = prostate differentiation factor = GDF-15	Absent	Up	
229868_s_at	PLAB = prostate differentiation factor = GDF-15	Absent		
221577_x_at	PLAB = prostate differentiation factor = GDF-15	Present no change		
<b>BMP related</b>				
<b>molecules</b>				
216047_x_at	SEZ6L, has BMP domains	Absent	Absent	<2
204926_at	Activin A = INHBA	Absent	Absent	<2
210141_s_at	INH A = Inhibin A	Present no change	Absent	<2
210511_s_at	INHBA	Absent	Absent	<2
205258_at	INHBB	Absent	Present no change	<3
207687_at	INHBC = Inhibin $\beta$ C	Present no change	Absent	<2
207688_s_at	INHBC = Inhibin $\beta$ C	Present no change	Absent	
210587_at	INHBE = Inhibin $\beta$ E	Present no change		<2
209591_s_at	TGF-B1	Down		
203084_at	TGF-B1	Absent	Present no change	4-7
220406_at	TGF-B2	Absent	Absent	<4
220407_s_at	TGF-B2	Absent		
209908_s_at	TGF-B2	Absent		
209909_s_at	TGF-B2	Absent		
209747_at	TGF-B3	Absent	Absent	Down
206012_at	TGF-B4 = EBAF = LEFTY	Absent	Absent	<2
221373_x_at	PSPN = persephin	Absent		<2
210683_at	NRTN = neurturin	Absent	Present no change	<2
230916_at	Nodal	Absent		



Table 1 Continued

Affymetrix probe ID		Significant change with BMP treatment in DU-145 cell line	Normal prostate vs. prostate cancer U-95A chip data	SAGE in prostate (tags per 200,000)
206268_at	LEFTB	Absent	Absent	
221359_at	GDNF	Absent		<2
206516_at	AMH = MIS	Absent	Absent	<2
<b>BMP receptors</b>				
203935_at	ALK-2, Genecard ACVR1	Present no change	Present no change	2-3
204832_s_at	BMPRI A	Present no change		
213578_at	BMPRI A = ALK-3	Present no change	Present no change	<2
210523_at	BMPRI B = ALK-6	Absent		
208223_s_at	BMPRI B = ALK-6	Present no change	Absent	<2
209920_at	BMPRI 2	Present no change	Absent	4-7
210214_s_at	BMPRI 2	Absent		
205327_s_at	ACVR2 = activin A receptor type II	Present no change	Absent	<2
220028_at	ACVR2B	Present no change	Absent	
208218_s_at	ACVR1B = ALK-4	Absent	Absent	2-3
208222_at	ACVR1B = ALK-4	Absent		
208219_at	ACVR1B = ALK-4	Absent		
206943_at	TGFBR1 = ALK-5	35% Present no change	Absent	
1552519_at	ACVR1C = ALK-7	Absent		
205209_at	Alk-4 = activin A receptor type IB	Absent		
204731_at	TGFBR3 = betaglycan	Present no change	Down	<2
226625_at	TGFBR3 = betaglycan	Present no change		
208944_at	TGFBR2	Present no change	Down	Down
207334_s_at	TGFBR2	Present no change		
210838_s_at	ACVRL1	Absent	Absent	<2
<b>BMP/TGF-<math>\beta</math> receptor interacting proteins/ downstream kinases</b>				
208756_at	EIF3S2 = TGF $\beta$ receptor interactin protein	Present no change	Unchanged	32-63
211536_x_at	MAP3K7 = TAK1	Present no change	Present no change	<2
211537_x_at	MAP3K7 = TAK1	Present no change		
206853_s_at	MAP3K7	Present no change		
206854_s_at	MAP3K7	Present no change		
218486_at	TIEG2	Present no change	Absent	2-3
216262_s_at	TGIF2	Present no change	Absent	
205210_at	TRAP-1	Absent	Absent	
228586_at	Endoglin = ENG	Absent		
201808_s_at	Endoglin = ENG	Absent	Present no change	<2
201809_s_at	Endoglin = ENG	Absent		
1563874_at	TRAG = WDR7	Absent	Present no change	2-3, up?
207644_at	FOXH1 = FAST1	Absent	Absent	
207530_s_at	CDKN2B	Absent	Absent	<2
221211_s_at	C21orf7	Present no change		<2
225557_at	AXUD1 = Tgf $\beta$ induced apoptosis protein	Present no change		4-7
202039_at	TIAF1 = Tgf $\beta$ induced anti-apoptotic factor 1	Present no change	Absent	4-7
201506_at	TGFB1 = BIGH3 = Tgf $\beta$ induced 1	Up	Present no change	Up
209622_at	STK16 = TGF- $\beta$ stimulated factor 1	Present no change	Absent	4-7
224938_at	SGK1	Present no change	Absent	<2
224939_at	SGK1	Present no change		
201739_at	SGK1	Present no change		
214044_at	RYR2	Absent		<2
207557_s_at	RYR2	Absent		
203901_at	TAB1 = MAP3K7IP1	Present no change	Absent	<2
<b>BMP signal transduction</b>				
210993_s_at	SMAD-1 = MADR1	Present no change	Present no change	<2
203075_at	SMAD-2	Present no change	Present no change	<2-6
203077_s_at	SMAD-2	Present no change		
203076_s_at	SMAD-2 = MADR2	Present no change		
205396_at	SMAD-3	Present no change	Present no change	<2-3
205398_s_at	SMAD-3	Present no change		
205397_x_at	SMAD-3 = JV15-2	Absent		
202527_s_at	SMAD-4	Present no change	Present no change	<2-9, down slightly
212347_x_at	SMAD-4	Present no change		
205187_at	SMAD-5	Present no change	Absent	<2
205188_s_at	SMAD-5	Present no change		
209887_at	SMAD-6	Absent		
209886_s_at	SMAD-6	Up		
213565_s_at	SMAD-6	Absent		
207069_s_at	SMAD-6	Up	Absent	<2-3
204790_at	SMAD-7	Up	Present, Down, $P = 0.036$	<2-3
206320_s_at	SMAD-9, =SMAD8A, SMAD8B, SMA9_Human	Absent	Absent	<2
220263_at	DAMS (Smad in the antisense direction)	Absent		<2
<b>SMAD interacting proteins</b>				
212668_at	SMURF-1	Absent	Absent	8-15
215458_s_at	SMURF-1	Present no change		
230820_at	SMURF-2	Up	Absent	<2-3, up slightly
205596_s_at	SMURF-2	U		
219409_at	SNIP = Smad nuclear interacting protein	Down		<2
203603_s_at	SIP-1 = ZFX1B = Smad interacting protein	Down	Absent	2-7, down
204893_s_at	MADHIP = SARA	Present no change	Present no change	<2
208446_s_at	MADHIP = SARA variant 2	Present no change		

Table 1 Continued

Affymetrix probe ID		Significant change with BMP treatment in DU-145 cell line	Normal prostate vs. prostate cancer U-95A chip data	SAGE in prostate (tags per 200,000)
203313_s_at	TGIF = TGIF_HUMAN	Present no change	Present no change	<2-7 up slightly
218724_s_at	TGIF2 = TGIF2_HUMAN	Present no change		<2
202160_at	CREBBP	Present no change	Present no change	8-15 to <2 down
211808_s_at	CREBBP	Absent		
218604_at	MAN1 = human homolog of SANE??	Present no change		
225426_at	MAN1 = human homolog of SANE??	Present no change		
225429_at	MAN1 = human homolog of SANE??	Present no change		
224886_at	CHIP = STUB1	Present no change		26-31
227625_s_at	CHIP = STUB1	Present no change		
233049_x_at	CHIP = STUB1	Present no change		
217934_x_at	CHIP = STUB1	Present no change		
202014_at	GADD34	Present no change	Present no change	<2
37028_at	GADD34	Present no change		
235629_at	DLX1 Distal-less homeobox 1	Absent		
1560100_at	DLX1 Distal-less homeobox 1	Absent		
211698_at	EID-1, =CRI1 gene card	Present no change	Present no change	32-63 to 16-32 down
1569080_at	Arkadia	Absent		<2
202811_at	AMSH	Present no change	Present no change	2-7, down slightly
231891_at	AMSH-2	Absent		<2
227607_at	AMSH-2	Present no change		
227606_s_at	AMSH-2	Present no change		
203304_at	NMA = Bambi	Up	Up	Up <2 to 8-15
218225_at	Ecsit = SITPEC	Present no change		Up <2 to 4-7
225498_at	TOB1	Present no change	Present no change	Up <2 to 16-31
228834_at	TOB1	Present no change		
204270_at	SKI	Present no change		
229265_at	SKI	Present no change	Absent	<2
213755_s_at	SKI	Absent		
215889_at	SNO = SKIL	Absent	Absent	<2
232379_at	SNO = SKIL	Absent		
200047_s_at	YY1	Present no change	Present no change	4-7
213494_s_at	YY1	Present no change		
201902_s_at	YY1	Absent		
205471_s_at	Dach1	Absent	Absent	Down slightly
205472_s_at	Dach1	Absent		

Abbreviation: TGF, transforming growth factor.

6-induced increase of noggin mRNA was not dependent on the presence of androgens (data not shown). The induction of noggin mRNA was observed in both the LNCaP (androgen receptor-positive) and DU-145 (androgen receptor-negative) cell lines, providing further evidence that this is an androgen-independent event (Fig. 2A).

The increased noggin production on BMP-6 treatment was also confirmed at the protein level. The addition of 100 ng/ml BMP-6 to DU-145 cell culture for 48 hours resulted in the expression of detectable amounts of noggin in the cells, whereas noggin protein was not detectable in cells cultured in the absence of BMP-6 (Fig. 1A, Lanes 3 and 4). Noggin protein was detected in the cells and their associated extracellular matrix, but not in the cell culture supernatant.

**Noggin Inhibits BMP-6 Activity.** Having shown that BMP-6 reproducibly up-regulates noggin at the mRNA and protein levels, we designed experiments to determine whether noggin could act as an antagonist to BMP-6. Previously, it had been shown that noggin inhibits the activity of BMP-2, -4, and -7, but the effect of noggin on BMP-6 had not been demonstrated. Increasing amounts of noggin were added to cells cultured in the presence of 100 ng/ml BMP-6 for 48 hours, and quantitative RT-PCR was used to measure the up-regulation of noggin mRNA. Noggin protein at higher doses (1-3  $\mu$ g/ml) reduced BMP-6 activity by 50% to 100% in both LNCaP and DU-145 cells. As a control, in the absence of BMP-6 protein, noggin protein had no discernible effect (Fig. 2C).

**BMP-6 Activates Negative Feedback Mechanisms.** Because elevated BMP-6 expression in prostate correlates with poor patient prognosis, we were interested in studying the expression of negative regulators of BMP activity. As described above, the profound effects of BMPs on cells and tissues have given rise to many levels of mechanisms to regulate these effects, including negative feedback loops that are activated by BMP-6. Table 2 is a summary of microar-

ray data showing that BMP-6 activates several negative feedback mechanisms when added to DU-145 cell culture. Inhibitory Smad-6 and Smad-7 are up-regulated and function to block BMP signal transduction by interfering with the activation of receptor Smads (Smads 1, 5, and 8; ref. 26). Smurf-2 is up-regulated by BMP-6 and functions by increasing the degradation rate of receptor Smads, thus attenuating BMP signaling (27). BAMBI was up-regulated by BMP-6 and acts as a kinase-deficient type I receptor unable to activate Smads (18). These proteins are examples of negative feedback regulation that reduce or weaken BMP signaling.

However, a few regulatory proteins were affected by BMP-6 that further activate the BMP signaling pathway. SIP-1, a Smad-binding protein and transcriptional repressor of E-cadherin, was down-regulated by BMP-6. E-cadherin has been proposed as a tumor suppressor gene, and its expression is frequently decreased in prostate cancers. SNIP-1 was also down-regulated by BMP-6 treatment and has been shown to suppress the transforming growth factor $\beta$  signaling pathway (28).

Again, we wanted to extend these findings beyond the DU-145 cell culture model and ask which of these BMP signaling genes are involved in prostate cancer. To a large extent, the DU-145 cancer cell model system agrees with both the SAGE and cancer *versus* normal prostate microarray data, although the data from the DU-145 cell arrays (Affymetrix 133 2.0 Plus chips) are much more complete than the chips used by Singh *et al.* (24) (Affymetrix U95A chips) and also more sensitive than the SAGE technology. The cancer *versus* normal prostate comparisons reveal information about BMP regulation beyond what is possible with DU-145 cell culture microarrays. For example, DAN, an extracellular BMP antagonist protein, was down-regulated in prostate cancers compared with normal prostate both on microarray and SAGE data sets and was present but not affected by

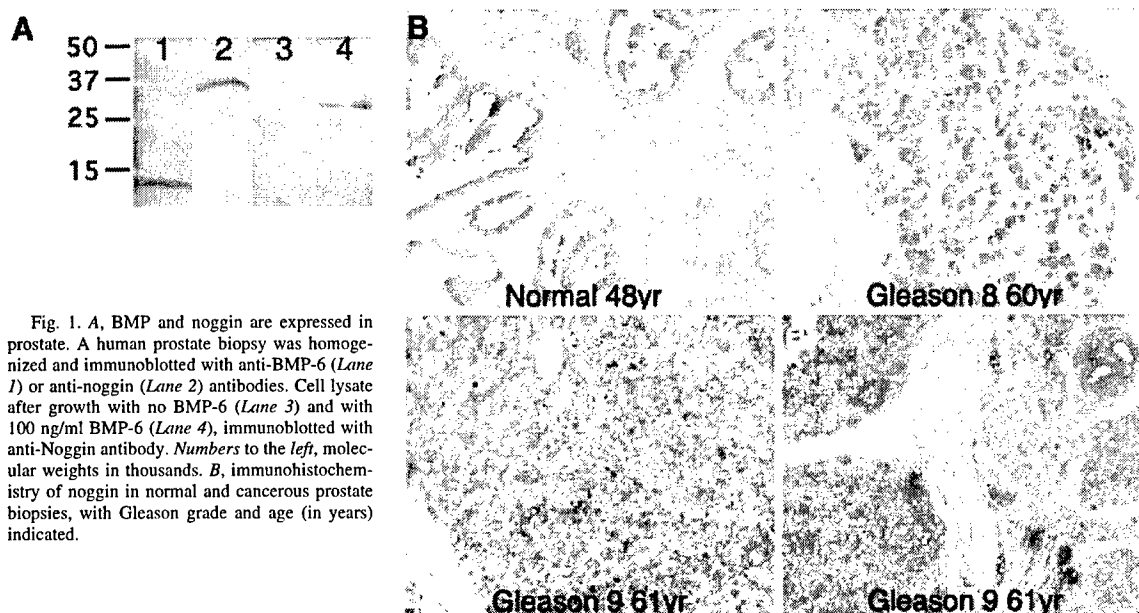


Fig. 1. A, BMP and noggin are expressed in prostate. A human prostate biopsy was homogenized and immunoblotted with anti-BMP-6 (Lane 1) or anti-noggin (Lane 2) antibodies. Cell lysate after growth with no BMP-6 (Lane 3) and with 100 ng/ml BMP-6 (Lane 4), immunoblotted with anti-Noggin antibody. Numbers to the left, molecular weights in thousands. B, immunohistochemistry of noggin in normal and cancerous prostate biopsies, with Gleason grade and age (in years) indicated.

BMP-6 in DU-145 cell culture. These comparisons are presented in Table 2.

In summary, we have demonstrated that BMP-6 induces noggin expression in prostate cancer cell culture at the mRNA and protein levels. Recombinant noggin protein inhibits this activity of BMP-6 in a prostate cancer cell culture system. In addition to noggin, BMP-6 activates several other negative (and some positive) feedback mechanisms that may attenuate BMP signal transduction. The cancer cell model data are in agreement with data derived from comparing normal and cancerous prostate.

**BMP-6 Induces Smad Phosphorylation.** Many studies have documented the function of BMPs in the context of bone development and embryogenesis. However, the function of BMP-6 in the adult prostate remains unknown. To extend the documented correlation

between elevated BMP-6 expression and tumor metastases, we wanted to (a) examine the BMP-6 signal transduction pathway, (b) identify some of the downstream targets, and (c) characterize the effects of BMP-6 on the biology of prostate cancer cells.

The first few steps in BMP signal transduction involve binding of BMP to its receptors, which are activated and subsequently phosphorylate Smads. These pathways are often disrupted in cancer cells (29). To test whether Smads are phosphorylated on BMP-6 treatment of DU-145 and LNCaP cells, immunoblots were performed on cell lysates and probed with antibodies that specifically recognize only phosphorylated Smad-1, -5, and -8. BMP-6 treatment induced Smad phosphorylation within 30 minutes, and elevated levels of phosphorylated Smads continued to be detected to at least 96 hours of BMP-6 treatment, as shown in Fig. 3A. All samples were compared with

Fig. 2. BMP-6 induces noggin, a BMP antagonist, and reduces proliferation. A, BMP-6 was added to DU-145 (□) and LNCaP (■) prostate cancer cell cultures for 48 hours, in the presence of 1% charcoal-stripped serum and 100 ng/ml DHT. Fold increase of noggin mRNA was measured by quantitative RT-PCR. B, increasing amounts of recombinant noggin were added to DU-145 cells for 48 hours in the presence of 100 ng/ml BMP-6 and 100 ng/ml DHT. BMP-6 activity was measured as increased noggin mRNA by quantitative RT-PCR. C, recombinant BMP-6 or noggin was added to cultures of DU-145 cells for 48 hours, and fold increase of noggin, gremlin, and cerberus mRNA was measured by quantitative RT-PCR. D, cells ( $2 \times 10^5$ ) were cultured for 7 days in a base medium of RPMI 1640 with 10% charcoal-stripped FBS and with the addition of either 100 ng/ml BMP-6, 5 nmol/L DHT, or both BMP-6 and DHT. DNA content measurement was done using PicoGreen.

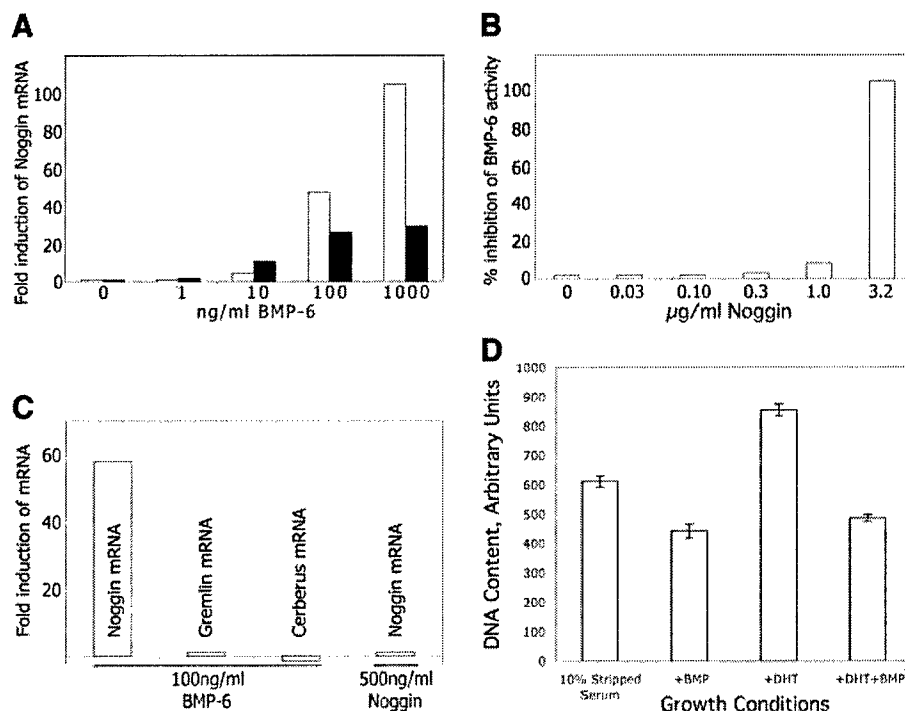


Table 2 BMP signaling molecules affected by BMP-6 in prostate cancer

Affymetrix probe ID	BMP related protein	Significant change with BMP treatment DU-145 cell line	U-95A chip publicly available prostate data (27 normal vs. 52 cancer)	SAGE in prostate (tags per 200,000)
231798_at	Noggin	Up	N/D	<2
201621_at	DAN = NBL	Present no change	Down	Down
221576_at	PLAB = prostate differentiation factor = GDF-15	Absent	Up	N/D
205258_at	INHBB	Absent	Present no change	<3
209591_s_at	TGF-B1	Down	Present no change	4-7
209747_at	TGF-B3	Absent	Down	Down
210683_at	NRTN = neurturin	Absent	Present no change	<2
204731_at	TGFBR3 = betaglycan	Present no change	Down	<2
208944_at	TGFBR2	Present no change	Down	Down
203304_at	NMA = Bambi	Up	Up	Up
228586_at	Endoglin = ENG	Absent	Present no change	<2
1563874_at	TRAG = WDR7	Absent	Present no change	2-3
201506_at	TGFB1 = BIGH3 = TGF- $\beta$ induced 1	Up	Present no change	Up
207069_s_at	SMAD-6	Up	Absent	<2-3
204790_at	SMAD-7	Up	Down? $P = 0.036$	<2-3
230820_at	SMURF-2	Up	Absent	<2-3
219409_at	SNIP = smad nuclear interacting protein	Down	N/D	<2
203603_s_at	SIP-1 = ZFHX1B = Smad interacting protein	Down	Absent	Down

Abbreviation: TGF, transforming growth factor; N/D, no data.

untreated cells cultured for the same length of time under otherwise identical conditions. The amount of unphosphorylated Smad-1 protein remained essentially identical between BMP-6-treated and control cells. Thus, the Smad phosphorylation pathway is intact in these cells and used for BMP signal transduction in response to exogenous BMP-6.

**BMP-6 Inhibits Cell Proliferation.** Although microarray analysis has provided insight into how cells respond to BMP-6 at the molecular level, the response at the cellular level is still unknown. One component of malignant prostate cancer is uncontrolled growth, and another component is androgen independence. Experiments were performed to determine whether BMP-6 affects the proliferation rate of androgen-dependent LNCaP cells. BMP-6 inhibited LNCaP cell proliferation after 1 week of cell culture in media supplemented with 10% charcoal-stripped (androgen-free) serum, as shown in Fig. 2D. Two lines of evidence indicated that this growth-inhibitory effect is not dependent on the presence of androgens. First, the effect was seen with or without addition of DHT to the medium. Second, the growth-inhibitory effect of BMP-6 was also seen in androgen-independent DU-145 cells (data not shown).

**BMP-6 Induces p21/CIP Expression.** The next set of experiments were done to gain insight into how BMP-6 may control cell proliferation. Microarray data showed that BMP-6 treatment caused a strong up-regulation of p21/CIP mRNA within 6 hours. P21/CIP is a cell cycle control protein shown to mediate BMP-dependent growth reduction in other cell types (30) and to be induced by BMP-2 and -4 in prostate (31).

Treatment with BMP-6 caused an increase of cell cycle control protein p21/CIP, which was detected at the mRNA level in DU-145 cells and confirmed at the protein level in both DU-145 and LNCaP cells. LNCaP cells expressed a higher basal level of p21 protein than DU-145 cells, and in both cell lines, there was a time-dependent increase of p21/CIP protein with BMP-6 treatment, as shown in Fig. 3B. P21/CIP protein levels were detectably increased after 90 minutes of BMP-6 treatment and remained elevated at least until 96 hours after BMP-6 treatment compared with cells cultured for the same length of time without BMP-6 in otherwise identical media. Thus we show evidence that the decreased proliferation of BMP-6-treated cells may be due in part to increased levels of p21/CIP protein.

A number of additional cell cycle control proteins were regulated at the mRNA level by BMP-6 treatment in DU-145 cells, as shown in Table 3. Most of these changes are consistent with reduced proliferation as a result of BMP-6 treatment. Cyclin-dependent kinase inhib-

itors p21/CIP, p18, and p19 were up-regulated, and cyclin-dependent kinases were down-regulated.

**BMP-6 Induces Expression of Id Proteins.** Id proteins are inhibitors of basic helix-loop-helix transcription factors that are under the control of BMP pathways in other cell types (32) and have been identified as markers of prostate cancer progression (33-35). We found that BMP-6 dramatically increased the expression of Id-1, Id-2, and Id-3 in the microarray, as shown in Table 3.

## DISCUSSION

The misregulation of BMP and BMP signaling pathways in prostate cancer is becoming increasingly apparent. BMP-6 was studied in this report due to the demonstrated correlation of increased prostatic BMP-6 expression and cancer severity. However, recent publications also show reduced expression of BMP-2 and Smads 4 and 8 in prostate cancers (29), and one report shows that BMP-5 is up-regulated in benign prostatic hyperplasia (36).

In this study, a comprehensive list of genes involved in generating and regulating the activities of BMPs was compiled. We tested the expression of these genes in DU-145 cells and analyzed how their

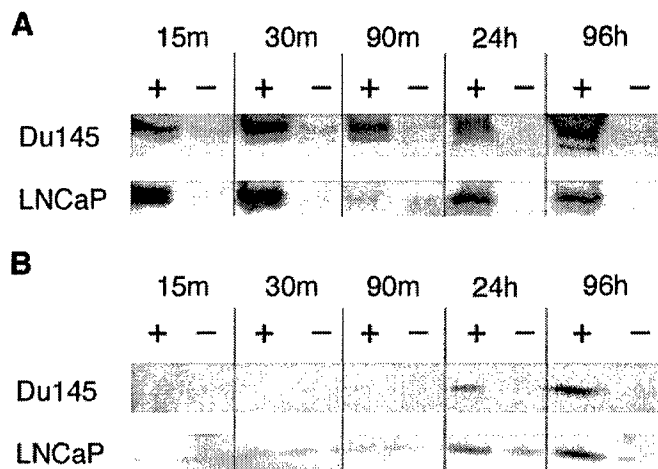


Fig. 3. BMP-6 increases Smad phosphorylation and p21 expression. Immunoblot of DU-145 and LNCaP cell lysates after treatment with 100 ng/ml BMP-6 for 15, 30, and 90 minutes; 24 hours; and 96 hours. Lysates from treated and untreated cells are at equivalent total protein in each sample. Primary antibody was anti-phospho-Smad in A; the membrane was stripped and reprobed with anti-p21 in B.

Table 3 Cell cycle and Id proteins regulated by BMP-6

Probe set	Gene	Accession no.	t Test
Up-regulated by BMP-6 treatment in DU-145 cells			
202284_s_at	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	NM_000389	0.0000
211792_s_at	Cyclin-dependent kinase inhibitor 2C (p18)	U17074	0.0468
210240_s_at	Cyclin-dependent kinase inhibitor 2D (p19)	U20498	0.0032
208501_at	Growth factor independent 1B (potential regulator of CDKN1A, translocated in CML)	NM_004188	0.0097
223394_at	CDK4-binding protein p34SEI1	BC002670	0.0138
210721_s_at	p21(CDKN1A)-activated kinase 7	AB040812	0.0489
208937_s_at	Inhibitor of DNA binding 1	D13889	0.0000
201565_s_at	Inhibitor of DNA binding 2	NM_002166	0.0000
207826_s_at	Inhibitor of DNA binding 3	NM_002167	0.0000
Down-regulated by BMP-6 treatment in DU-145 cells			
203154_s_at	p21(CDKN1A)-activated kinase 4	NM_005884	0.026
239470_at	p21(CDKN1A)-activated kinase 6	AI668644	0.028
1555310_a_at	p21(CDKN1A)-activated kinase 6	BC035596	0.037
209112_at	Cyclin-dependent kinase inhibitor 1B (p27, Kip1)	BC001971	0.009
219534_x_at	Cyclin-dependent kinase inhibitor 1C (p57, Kip2)	NM_000076	0.027
216894_x_at	Cyclin-dependent kinase inhibitor 1C (p57, Kip2)	D64137	0.022
213348_at	Cyclin-dependent kinase inhibitor 1C (p57, Kip2)	N33167	0.035
203468_at	Cyclin-dependent kinase (CDC2-like) 10	NM_003674	0.015

NOTE. Accession numbers refer to GenBank and RefSeq entries (<http://www.ncbi.nlm.nih.gov/>).

expression is regulated by BMP-6. To relate these cell culture observations to whole prostates, we searched publicly available data from National Center for Biotechnology Information and Massachusetts Institute of Technology, and we found that the cell culture model in many ways mimics what occurs in the prostate. Of the top 50 genes with high expression in tumor and the top 50 genes with high expression in normal prostate identified by Singh *et al.* (24), 13 are either up- or down-regulated by BMP-6 in DU-145 cell culture. Comparisons between cell culture and whole prostate microarray data, although useful to extend the validity of cell culture observations, must also be viewed critically because of the many cell types contributing to RNA from whole prostates.

Special attention was paid to antagonists of BMP activity, especially noggin. Prostate cancer cells were shown to express BMP-6 and noggin at the mRNA and protein levels. Noggin was localized predominantly in normal epithelial and carcinoma cells in prostate tissue biopsies. Surprisingly, it was not detected in cell culture unless the cells were stimulated with BMP-6. Together with the observation that noggin was not detectable in the cell culture supernatant, this may indicate that there is very limited diffusion of the protein away from the cells in which it is produced. It will also be interesting to study whether the BMP-6 produced by prostate carcinoma can diffuse past the noggin bound to the heparan sulfate proteoglycan in the extracellular matrix (37, 38). Because little is known about the relative binding affinities of noggin to the various BMP family members, it remains to be determined whether noggin specifically inhibits BMP-6 availability in prostate epithelium or whether it functions as a general antagonist of all BMPs.

BMP-6 inhibited the proliferation of DU-145 and LNCaP cells. We demonstrated that BMP-6 causes changes in the expression of cell cycle control proteins. Specifically, BMP-6 caused strong up-regulation of p21/CIP, which may explain in part the reduced proliferation seen in BMP-6-treated cells. This effect was not androgen dependent because BMP-6 reduced proliferation and increased p21/CIP expression in both DU-145 and LNCaP cells, and in the presence and absence of DHT. DU-145 cells, in addition to being androgen independent, have a missense mutation in the p53 gene (39). This indicates that BMP-6 induces p21/CIP protein and mRNA expression in a p53-independent manner.

The antiproliferative response of prostate cancer cells to BMP-6 seems counterintuitive, given the established correlation between aggressive cancer and elevated BMP-6 expression. However, the BMP-

6-induced increase in Id protein expression may explain in part the increased resistance to apoptosis (40) and the correlation between BMP overexpression and cancer progression. In prostate cancers metastatic to bone, the effect of the BMP-6 in the surrounding bone environment may be related to the osteosclerotic nature of the metastatic lesions.

The findings that BMP-6 up-regulated noggin expression at mRNA and protein levels and, conversely, that noggin inhibited BMP-6 function have potential therapeutic implications for noggin in ameliorating prostate bone metastases and attendant pain and morbidity. The challenge for the future is to dissect BMP-6 signaling in prostate cancer, both in the prostate cancer in situ and in the bone microenvironment in the immediate vicinity of the metastases.

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